

Research article

THE INFLUENCE OF LTS-4, A SAPONOSIDE FROM *Lysimachia thyrsoflora* L., ON HUMAN SKIN FIBROBLASTS AND HUMAN MELANOMA CELLS

AGNIESZKA GALANTY^{1*}, MARTA MICHALIK², ŁUKASZ SĘDEK²
and IRMA PODOLAK¹

¹Department of Pharmacognosy, Faculty of Pharmacy, Jagiellonian University, ul. Medyczna 9, 30-688 Kraków, Poland, ²Department of Cell Biology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, ul. Gronostajowa 7, 30-387 Kraków, Poland

Abstract: We investigated the effect of a triterpene saponoside from *Lysimachia thyrsoflora* L. upon the viability, proliferation, morphology and cell motility of human melanoma HTB-140 cells and human skin fibroblasts (HSFs). The compound, denoted LTS-4, decreased the viability and rate of cell growth of both cell types in a time- and dose-dependent manner, and proved cytotoxic against cancer cells at significantly lower concentrations than for fibroblasts. LTS-4 also affected the morphology of the examined cells, causing vacuolisation and actin cytoskeleton disintegration, and had an inhibitory effect on the tumour cell motility.

Key words: Triterpene saponoside, Cytotoxicity, *Lysimachia thyrsoflora* L., Melanoma, Fibroblasts

INTRODUCTION

Triterpenoid saponins are one of the most frequently occurring groups of secondary metabolites. They are characterized by a wide range of pharmacological activities. Numerous reports describe their cytotoxic and

* Author for correspondence: e-mail: mfgalant@cyf-kr.edu.pl

Abbreviations used: CME – coefficient of movement efficiency; PBS – phosphate buffered saline; TLC – thin-layer chromatography; TRITC phalloidin – phalloidin-conjugated tetramethylrhodamine B isothiocyanate

antiproliferative properties. A triterpene saponin isolated from the root bark of *Aralia dasyphylla* showed significant cytotoxic activity against human epidermal carcinoma (KB) cells and human negroid cervix carcinoma (HeLa-S3) cells, with IC₅₀ values of 1.2 µg/ml for KB cells and 0.02 µg/ml for HeLa-S3 cells [1]. Hederacolchiside A1, an oleanolic acid monodesmoside from *Hedera colchica*, showed strong cytotoxic and antiproliferative activities against a number of cancer cells, with an IC₅₀ between 4.5 and 12 µM [2]. Marquina *et al.* reported that a mixture of monodesmoside saponins, which were not active as pure single compounds, was highly cytotoxic against mouse leukemia (P388) and colon cell lines, with respective ED₅₀ values of 2.3 and 3.6 µg/ml [3].

As part of our search to identify naturally occurring anticancer agents, we found that the methanolic extract from the underground parts of *Lysimachia thyrsiflora* L. (*Primulaceae*) showed a significant cytotoxicity against sarcoma XC cells, and this extract was selected for bioactivity-guided fractionation [4]. Previous phytochemical studies of *L. thyrsiflora* resulted in the isolation of flavonoids and saponins from the aboveground parts of the plant [5-7]. Our research on the underground parts of *L. thyrsiflora* led to the isolation of a number of triterpene glycosides. One of compounds, denoted LTS-4, showed promising cytotoxic activity in a preliminary screening, and was characterized chemically as 3-O- $\{\beta$ -D-xylopyranosyl-(1 \rightarrow 2)- β -D-glucopyranosyl-(1 \rightarrow 4)- $[\beta$ -D-glucopyranosyl-(1 \rightarrow 2)]- α -L-arabinopyranosyl}-cyclamiretin A [8]. The cytotoxic activity of LTS-4 against murine B16 and XC cell lines was studied previously [submitted, 2007], and in this paper, we report the results of *in vitro* assays for the cytotoxicity of LTS-4 against human cell lines.

MATERIALS AND METHODS

Plant material

The underground parts of authenticated specimens of *Lysimachia thyrsiflora* L. were cultivated in the Garden of Medicinal Plants at the Faculty of Pharmacy, Jagiellonian University, Kraków, Poland. A voucher specimen is deposited at the Department of Pharmacognosy, Faculty of Pharmacy, Jagiellonian University, Kraków. The plant material was collected in September and air-dried.

Plant extraction and isolation of LTS-4

The dried, crushed plant material was extracted successively with chloroform and methanol with the addition of 0.5% pyridine on a water bath. The methanol extract was evaporated to dryness and the residue was dissolved in water and eluted with n-buthanol. The elution was repeated until the water phase showed no positive reaction for saponins (TLC). The buthanol eluates were combined and evaporated to dryness under reduced pressure. The crude buthanol extract was subjected to repeated column chromatography on silica gel (chloroform-methanol-water 23:12:2) followed by preparative TLC on silica-gel plates to obtain LTS-4. The compound was recrystallized from methanol and characterised chemically based on 1D, 2D NMR and MS data.

Cell culture

Human skin fibroblasts (HSFs) and human melanoma HTB140 cells were used. HSFs were obtained from skin grafts of adult, healthy donors and cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS), 100 IU/ml penicillin and 10 µg/ml streptomycin. The Hs 294T human melanoma cell line (No. HTB140) was obtained from the American Type Culture Collection (ATCC, Rockville, USA). The cells were cultivated as described in [9]. All the cells were cultured in a humidified atmosphere with 5% CO₂ at 37°C.

Triterpene glycoside treatment

Stock solutions of LTS-4 were diluted in the culture medium to final concentrations of 0-35 µg/ml with a final methanol concentration of 0.1%. HSFs and HTB140 cells were incubated with or without LTS-4 in the culture medium for different time periods (from 1 to 10 days).

Cytotoxic assay

Cells were seeded onto 24-well plates at 1.5×10^4 cells per well. 24 h after seeding, the culture medium was replaced with the same medium containing different concentrations of LTS-4 (from 0 up to 35 µg/ml). The controls were incubated in the culture medium with 0.1% methanol. All the cells were incubated at 37°C for 24, 72 or 144 h, and then cell viability was determined by the Trypan blue exclusion dye test or by the fluoresceine diacetate and ethidium bromide test [10].

Measurement of cell proliferation

Cells were seeded onto 24-well plates at an initial density of 3×10^3 cells/cm², and incubated for 24 h at 37°C. The medium was replaced with fresh culture medium (control) or with the same medium containing different concentrations of LTS-4 (from 0 to 35 µg/ml). After various incubation times (24, 48, 72, 96, 120 and 144 h) the medium was removed, the cells were harvested from the plate wells by trypsinization, and the number of cells was determined using a Bürker's chamber.

Staining of the actin cytoskeleton

To visualize the actin cytoskeleton, the cells were grown on glass coverslips inserted into 12-well plates, in the culture medium without or with 5 or 10 µg/ml LTS-4 for 24, 48 or 96 h. Then the cells were washed with PBS and fixed in 3.7% formaldehyde in PBS for 15 min at 37°C. After washing 3 times with PBS, the cells were permeabilized in 0.1% Triton X-100 solution for 15 min, and the F-actin was stained with TRITC-phalloidin at a concentration of 500 ng/ml for 45 min. Fluorescent and Nomarski's differential interference contrast images were taken with a Leica DM IRE-2 microscope.

Time lapse-monitoring of the movement of individual cells

Cell behaviour was observed with an inverted Olympus IMT-2 microscope with phase contrast optics using a 10x objective at 37°C. HTB-140 cells were plated to a Corning flask at a density of 2×10^4 cells/cm², and incubated in DMEM supplemented with 10% FCS for 24 h. Then the medium was replaced with fresh culture medium (control) or with the same medium containing 5 or 10 µg/ml of LTS-4, and cell movement was recorded for 8 h. The tracks of individual cells were generated as described previously [11, 12]. The cell trajectories were constructed from 88 subsequent cell centroid positions recorded over 440 min at 5-min time intervals, in the control medium, immediately after the addition of LTS-4 and 24 h after the addition of this saponoside. The cell trajectories were presented in circular diagrams with the starting point of each trajectory situated at the diagram center [12-14].

For the analysis of the parameters characterizing cell locomotion, the “Mathematica” program by Stephen Wolfram was used.

The following parameters characterizing cell locomotion were computed for each cell:

- the total length of the cell trajectory (µm);
- the total length of the cell displacement from the starting point to the final position of the cell (µm);
- the coefficient of movement efficacy (CME), defined as the ratio of cell displacement to cell trajectory length;
- the average speed of cell movement (µm/min);
- the average speed of cell displacement (µm/min).

Statistical analysis

For each value measured, 45-300 cells were analysed. Each variable was expressed as the mean (\pm SEM). The statistical significance was determined using the Student's t-test or the non-parametric Mann-Whitney *U*-test, with $p < 0.05$ considered to indicate significant differences. For morphological and fluorescent staining evaluation, at least 20 microscopic fields of view were analysed.

RESULTS**The effect of LTS-4 on the viability and growth of HSFs and melanoma HTB-140 cells**

In order to evaluate the effect of LTS-4 on normal human skin fibroblasts and cancer melanoma HTB 140 cells, microscopic observations and cytotoxic assays were performed. The trypan blue exclusion dye test and the fluoresceine diacetate and ethidium bromide test were applied to examine the viability of cells incubated without or with LTS-4 for different periods. The results obtained by both methods were similar, without statistically significant differences. The data for the trypan blue test shown in Tab. 1 indicates that the viability of the two cell lines incubated in the presence of LTS-4 was strongly dependent on its

concentration in the culture medium, and that the cytotoxic activity of LTS-4 was higher against HTB-140 cells than against HSFs. At 5 $\mu\text{g/ml}$, LTS-4 had no significant effect on the viability of HSFs ($95.2 \pm 4.9\%$ and $93.7 \pm 1.1\%$ cells still alive after 24 and 144 h treatment, respectively) but significantly decreased the viability of melanoma cells ($85.1 \pm 5.8\%$ and $67.6 \pm 6.5\%$ cells still alive after 24 and 144 h treatment, respectively). The cytotoxic activity against HTB-140 cells was also more time-dependent than for HSFs. The decrease in LTS-4-induced cell viability was reversible in fibroblasts at concentrations up to 35 $\mu\text{g/ml}$, but only at low concentrations (up to 10 $\mu\text{g/ml}$) in tumour melanoma cells (data not shown).

Tab. 1. The effect of LTS-4 on the viability of human skin fibroblasts (HSFs) and melanoma HTB-140 cells.

LTS-4 ($\mu\text{g/ml}$)	Viability of cells \pm SE (%)					
	HSF			HTB-140		
	24 h	72 h	144 h	24 h	72 h	144 h
0	98.6 ± 1.5	98.1 ± 0.7	99.3 ± 1.4	96.0 ± 5.5	92.9 ± 2.4	93.13 ± 6.2
5	95.2 ± 4.9	94.4 ± 3.5	$93.7 \pm 1.1^*$	$85.1 \pm 5.8^*$	$75.8 \pm 8.7^*$	$67.6 \pm 6.5^*$
10	$89.4 \pm 3.5^*$	$88.5 \pm 3.1^*$	$81.4 \pm 1.2^*$	$79.1 \pm 2.7^*$	$66.3 \pm 7.4^*$	$48.9 \pm 3.9^*$
15	$81.2 \pm 1.2^*$	$76.3 \pm 3.5^*$	$71.0 \pm 0.8^*$	$73.7 \pm 4.1^*$	$36.2 \pm 6.9^*$	$21.5 \pm 0.7^*$
25	$68.3 \pm 3.4^*$	$40.2 \pm 3.5^*$	$18.1 \pm 3.5^*$	$50.1 \pm 11.4^*$	$33.1 \pm 3.0^*$	$14.4 \pm 4.2^*$
35	$59.7 \pm 1.0^*$	$36.9 \pm 2.3^*$	$10.5 \pm 3.2^*$	$38.8 \pm 4.0^*$	$19.1 \pm 3.2^*$	$0.0 \pm 0.0^*$

Cells were grown in DMEM supplemented with 10% foetal calf serum without (0 $\mu\text{g/ml}$) or with LTS-4 (from 5 up to 35 $\mu\text{g/ml}$), for 24, 72 or 144 h. Cell viability was determined using the trypan blue exclusion dye test. The values are the means of the number of living cells (%) for at least 300 cells for each experimental point. The results presented are the means of three separate experiments. The values significantly different from the control are indicated by a *.

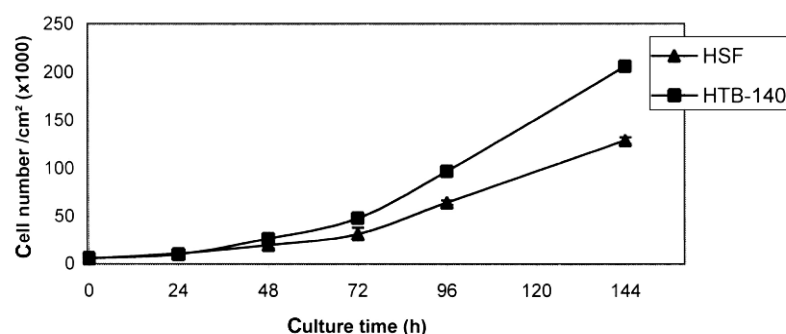


Fig. 1. The proliferation of human skin fibroblasts (HSFs) and human melanoma (HTB140) cells under control conditions. Cells were seeded onto 24-well plates at an initial density of 3×10^3 cells per well, and grown in DMEM supplemented with 10% foetal calf serum. The number of cells was determined with the use of a Bürker's chamber. The cells were counted every 24 h. Each experiment was performed twice in duplicate.

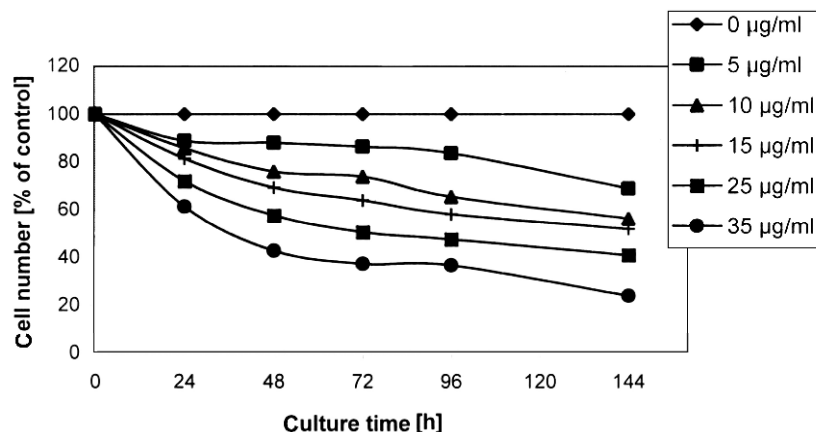


Fig. 2. The effect of LTS-4 on the proliferation of human skin fibroblasts. Cells were seeded onto 24-well plates at an initial density of 3×10^3 cells per well, and grown in DMEM supplemented with 10% foetal calf serum without (0 µg/ml) or with LTS-4 (from 5 up to 35 µg/ml) for 144 h. The number of cells was determined with the use of a Bürker's chamber. The cells were counted every 24 h. Each experiment was performed twice in duplicate and the results were expressed as the cell number compared to the control.

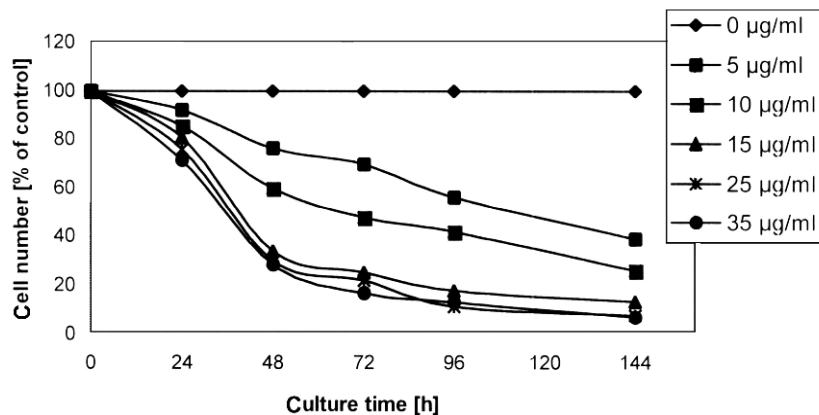


Fig. 3. The effect of LTS-4 on the proliferation of melanoma HTB-140 cells. Cells were seeded onto 24-well plates at an initial density of 3×10^3 cells per well, and grown in DMEM with 4.5 g/l glucose supplemented with 10% foetal calf serum without (0 µg/ml) or with LTS-4 (from 5 to 35 µg/ml) for 144 h. The number of cells was determined with the use of a Bürker's chamber. The cells were counted every 24 h. Each experiment was performed twice in duplicate, and the results were expressed as the cell number compared to the control.

In the next series of experiments, the growth of the two cell types used in these studies was examined under different culture conditions. Fig. 1 shows the rate of proliferation of human skin fibroblasts in comparison to that of human

melanoma cells (HTB140) under control conditions. The growth rates of both cell lines were exponential, and the greater acceleration of cell growth was observed in the melanoma cell culture. Figs 2 and 3 respectively show the effect of LTS-4 on the proliferation of fibroblasts and melanoma HTB-140 cells. It was found that LTS-4 inhibited the proliferation of both investigated cell lines in a time- and dose-dependent manner. Human melanoma cells were more sensitive to the presence of LTS-4 and their proliferation was strongly concentration dependent. After 48 h incubation at 5 $\mu\text{g/ml}$, significant differences between the growth of HSFs and HTB-140 cells were observed. The differences became more profound with longer incubation time.

The effect of LTS-4 on the morphology of HSFs and melanoma HTB-140 cells

The effect of LTS-4 on the morphology of HSFs and HTB-140 cells was observed by microscopic examination. The tested compound caused morphological changes in both cell types in a time- and dose-dependent manner. The saponoside at a concentration of 5 $\mu\text{g/ml}$ did not induce cell shape changes in HSFs (Fig. 4C, D). HSFs treated with 10 $\mu\text{g/ml}$ of LTS-4 for 24 h or longer retracted or rounded and formed numerous blebs instead of lamellipodia (Fig. 4E, F). In contrast to the fibroblast cells, HTB-140 cells were significantly more vulnerable to LTS-4. The incubation of HTB-140 cells for 24 h in the presence of 5 $\mu\text{g/ml}$ of LTS-4 resulted in the contraction of the cells (Fig. 4C, D).

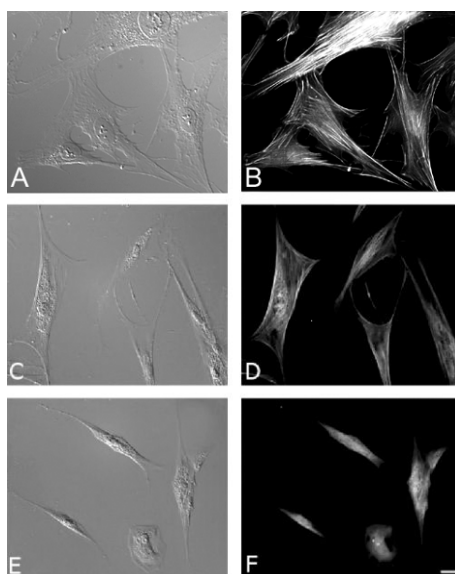


Fig. 4. The effect of LTS-4 on the morphology and actin cytoskeleton of human skin fibroblasts. Cells were grown in DMEM supplemented with 10% foetal calf serum without (A, B) or with 5 $\mu\text{g/ml}$ (C, D) or 10 $\mu\text{g/ml}$ (E, F) for 24 h. Microphotographs were taken under an inverted LEICA DMIRE2 microscope, with Nomarski's differential interference contrast optics (A, C, E) and with epifluorescence (B, D, F). Scale bar = 25 μm .

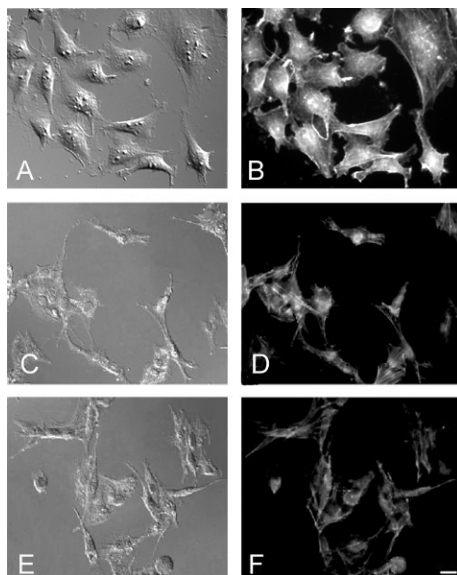


Fig. 5. The effect of LTS-4 on the morphology and actin cytoskeleton of melanoma HTB-140 cells. Cells were grown in DMEM with 4.5 g/l glucose supplemented with 10% foetal calf serum without (A, B) or with 5 $\mu\text{g/ml}$ (C, D) or 10 $\mu\text{g/ml}$ (E, F) LTS-4 for 24 h. Microphotographs were taken under an inverted LEICA DMIRE2 microscope, with Nomarski's differential interference contrast (A, C, E) and with epifluorescence (B, D, F). Scale bar = 25 μm .

It was also observed that LTS-4-treated melanoma cells were less adherent to the substratum. In both LTS-4-treated cell lines, vacuolization of cytoplasm was also noted (Figs 4 and 5).

The effect of LTS-4 on the actin cytoskeleton of HSFs and melanoma HTB-140 cells

An examination of the actin cytoskeleton organization by fluorescence microscopy revealed differences between the LTS-treated and control cells. HSFs grown in the control medium displayed well-developed bundles of F-actin (stress fibres) arranged parallel to the long axis of the cell (Fig. 4B). Incubating fibroblasts in the medium containing 5 $\mu\text{g/ml}$ LTS for 24 hours or longer induced a disappearance of some stress fibres and a reorganization of the F-actin network to intensely fluorescent patches which were randomly distributed throughout the cytoplasm (Fig. 4D).

HSFs treated with 10 $\mu\text{g/ml}$ of the tested compound were characterized by a loss of stress fibres, and F-actin accumulation around the nucleus (Fig. 4F). Melanoma HTB-140 cells treated with 5 $\mu\text{g/ml}$ or 10 $\mu\text{g/ml}$ LTS differed with respect to the structure and distribution of F-actin as compared to the control cells (Fig. 5). The control cells showed some F-actin bundles and cytoplasmic F-actin staining (Fig. 5B). LTS-4 at both examined concentrations caused the disruption of F-actin after 24 h of incubation or longer (Fig. 5D, F).

The effect of LTS-4 on the motile activity of human skin fibroblasts and melanoma HTB-140 cells

The ability of LTS-4 to affect the HSF and HTB-140 cell motility was examined as the last part of this study. Fig. 6 shows the trajectories of fibroblasts moving in the culture medium supplemented with 10% of FCS without (Fig. 6A) or with 5 $\mu\text{g/ml}$ (Fig. 6B) or 10 $\mu\text{g/ml}$ (Fig. 6C) LTS-4, or after 24 h preincubation with the saponoside (Fig. 6D). The quantitative analysis of the individual tracks (Tab. 2) shows that LTS-4 has a concentration- and time-dependent effect on the motility of human skin fibroblasts.

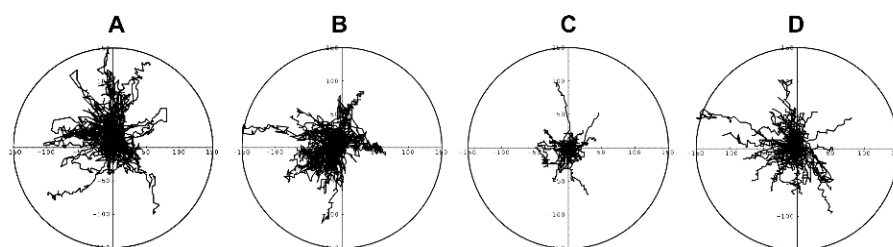


Fig. 6. The effect of LTS-4 on the motile activity of human skin fibroblasts. Composite trajectories of human skin fibroblasts migrating in DMEM supplemented with 10% foetal calf serum (control, A), in the medium containing 5 $\mu\text{g/ml}$ LTS-4, immediately after the addition of the saponoside (B), in the medium containing 10 $\mu\text{g/ml}$ LTS-4, immediately after the addition of the saponoside (C), and in the medium containing 5 $\mu\text{g/ml}$ LTS-4 24 h after the addition of the saponoside (D), displayed in circular diagrams drawn with the initial point of each trajectory placed at the origin of the plot. The panels show the trajectories of 45 individual cells. Axis scale in μm .

Tab. 2. Summary of the quantitative data showing the effect of LTS-4 on the motility of human skin fibroblasts immediately and 24 h after saponoside administration.

Movement parameters	LTS-4 concentration ($\mu\text{g/ml}$)			
	0	5	10	5 (after 24 hours)
Total length of cell trajectory (μm)	337.3 \pm 12.1	373.1 \pm 13.4	111.1 \pm 53*	179.4 \pm 13*
Average speed of cell movement ($\mu\text{m/min}$)	0.77 \pm 0.02	0.85 \pm 0.01	0.25 \pm 0.01*	0.41 \pm 0.03*
Total length of cell displacement (μm)	54.8 \pm 4.8	44.4 \pm 3.99	10.6 \pm 1.4*	50.4 \pm 4.9
Average rate of cell displacement ($\mu\text{m/min}$)	0.13 \pm 0.01	0.10 \pm 0.01	0.02 \pm 0.01*	0.11 \pm 0.01
Coefficient of movement efficiency (CME)	0.17 \pm 0.02	0.12 \pm 0.01	0.09 \pm 0.01*	0.34 \pm 0.03*

The average speed of cell movement is defined as the total length of cell trajectory/time of recording (440 min). The average rate of cell displacement is defined as the total length of cell displacement from the starting point to the final cell position/time of recording (440 min). The coefficient of movement efficiency is the ratio of cell displacement to cell trajectory length. The CME would equal 1 for a cell moving persistently along one straight line in one direction and 0 for random movement. The values are means \pm S.E.M. * significant at $p < 0.05$ vs. the control.

The trajectories of the melanoma HTB-140 cells moving under the control conditions and in the presence of 5 and 10 $\mu\text{g/ml}$ LTS-4 are shown in the circular diagrams in Fig. 7. The motile activity of cells treated with LTS-4 was analysed both immediately after the addition of LTS-4 and after 24 h preincubation with 10 $\mu\text{g/ml}$ LTS-4 saponoside. The quantitative data obtained is summarized in Tab. 3.

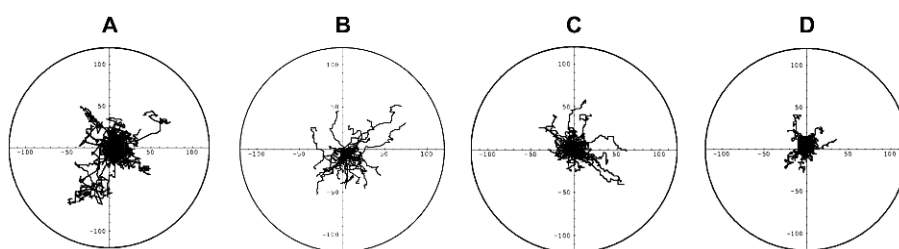


Fig. 7. The effect of LTS-4 on the motile activity of melanoma HTB-140 cells. Composite trajectories of melanoma HTB-140 cells migrating in DMEM with 4.5 g/l glucose supplemented with 10% foetal calf serum (control, A), in the medium containing 5 $\mu\text{g/ml}$ LTS-4, immediately after the addition of the saponoside (B), in the medium containing 10 $\mu\text{g/ml}$ LTS-4, immediately after the addition of the saponoside (C) and in the medium containing 10 $\mu\text{g/ml}$ LTS-4, 24 h after the addition of the saponoside (D), displayed in circular diagrams drawn with the initial point of each trajectory placed at the origin of the plot. The panels show the trajectories of 45 individual cells. Axis scale in μm .

Tab. 3. Summary of the quantitative data showing the effect of LTS-4 on the motility of human melanoma HTB-140 cells immediately and 24 h after saponoside administration.

Movement parameters	LTS-4 concentration ($\mu\text{g/ml}$)			
	0	5	10	10 (after 24 h)
Total length of cell trajectory (μm)	192.6 ± 10.4	$133.6 \pm 6.8^*$	$91.3 \pm 3.1^*$	$105.4 \pm 3.5^*$
Average speed of cell movement ($\mu\text{m/min}$)	0.44 ± 0.02	0.31 ± 0.02	$0.21 \pm 0.01^*$	$0.24 \pm 0.01^*$
Total length of cell displacement (μm)	25.5 ± 2.5	29.4 ± 2.8	21.9 ± 2.6	$12.6 \pm 1.5^*$
Average rate of cell displacement ($\mu\text{m/min}$)	0.06 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	$0.03 \pm 0.01^*$
Coefficient of movement efficiency (CME)	0.14 ± 0.01	0.22 ± 0.02	0.23 ± 0.02	0.12 ± 0.01

The average speed of cell movement is defined as the total length of cell trajectory/time of recording (440 min). The average rate of cell displacement is defined as the total length of cell displacement from the starting point to the final cell position/time of recording (440 min). The coefficient of movement efficiency is the ratio of cell displacement to cell trajectory length. The CME would equal 1 for a cell moving persistently along one straight line in one direction and 0 for random movement. The values are means \pm S.E.M. * significant at $p < 0.05$ vs. the control.

It was found that LTS-4 inhibited the motile activity of melanoma HTB-140 cells during the first 8 h of the treatment. A statistically significant decrease in

the average speed of cell movement was observed but not in the total cell displacement. Exposing cells to 10 µg/ml of LTS-4 for a longer time (24-32 h) caused a decrease in both the average speed of locomotion (to 55% of the control) and the average rate of cell displacement (to 50% of the control; see Tab. 3). Moreover, it was observed that the HTB-140 cells migrated in all directions with the same probability (Fig. 7).

DISCUSSION

This study was undertaken to evaluate the cytotoxic and antiproliferative effect of a triterpene glycoside, LTS-4, isolated from the underground parts of *Lysimachia thyrsoiflora*, on two human cell lines: skin fibroblasts (HSFs) and melanoma HTB140 cells. The effect of LTS-4 on cell morphology, the actin cytoskeleton and cell motility was also studied. The results indicate that LTS-4 decreased the viability and rate of cell growth of both cell types in a dose- and time-dependent manner. The human fibroblasts cells seemed to be less sensitive to the cytotoxic effect of LTS-4 than the human melanoma HTB140 cells (Tab. 1, comp. Figs 2 and 3). The decrease in the viability and inhibition of proliferation induced by LTS-4 was reversible in HSF cells across a broad spectrum of concentrations, but only at concentrations below 10 µg/ml in HTB140 cells. This effect may be due to the characteristic ability of saponins to bind with sterols, specifically with the membrane cholesterol [15, 16], and the resulting permeabilization of the cell membrane. Galeotti *et al.* [17] showed that the cholesterol content in hepatoma cell microsomal and plasma membranes is much higher than in normal cells. Significant differences in the lipid content in human healthy and neoplastic renal tissues were also described by Tugnoli *et al.* [18]. This may provide one possible explanation for the higher cytotoxic effect of LTS-4 on HTB140 cells.

LTS-4 affected not only the viability and growth rate of the tested cell lines, but also their cell morphology. It caused the contraction of HSFs and melanoma cells in a dose- and time-dependent manner, and in the final effect, the cells became less adherent to the substratum. The compound changed the size and shape of both cell types, causing cytoplasm vacuolisation even at low concentrations, and the effect was more profound in fibroblast cells. A similar vacuolisation effect was observed in the same HSFs in our previous studies with some local anaesthetics [19, 20]. We showed that the cytoplasm vacuolisation was caused by changes in fluid-phase endocytosis and exocytosis, and was connected with actin cytoskeleton disintegration, probably due to the actomyosin contraction. The mechanism of LTS-4-induced vacuolisation and cytoskeleton disintegration may be analogical, but further study is needed.

In both cell types, there was an observable influence of LTS-4 on cytoskeleton organization. The compound caused the disintegration of the actin cytoskeleton in HSFs and HTB140 cells (a loss of stress fibres was observed) dependent on incubation time and the concentration of LTS-4 in the culture medium (Fig. 4).

The actin cytoskeleton is engaged in setting cell shape, and the formation of structures like lamellipodia, filopodia and stress fibres [21, 22], and it also affects the flattening and attachment of the cell to the substratum [23]. Thus, LTS-4-induced changes in the organisation of F-actin in the examined cells might be the reason for its influence on the cell morphology and motility. Baumann *et al.* [24] observed that the saponin-cell membrane interaction caused not only the permeabilization of the latter but also transmembrane protein (ion channels, integrins) complexation in a defined position of the membrane, forcing changes in cytoskeleton organization [24]. These membrane protein rearrangement-induced cytoskeleton changes might be one of the possible mechanisms of LTS-4 activity.

As the changes in cytoskeleton organisation might influence cell motility, our experiment also aimed to examine whether LTS-4 affects the movement of HSFs and HTB140 cells. Some other saponins, isolated from ginseng root or soybean, were observed to suppress the metastatic potential of murine B16 melanoma cells *in vitro* and *in vivo* [25-27]. In our experiment, LTS-4 influenced the movement of human skin fibroblasts directly after its addition to the culture medium at a concentration of 10 µg/ml, causing statistically significant changes in the average length of the cell trajectory value in comparison to the control (Tab. 2). The lowest tested concentration did not influence the cell motility statistically, but 24 h preincubation with the tested compound caused the cells to begin moving again, and the changes in the cell movement values indicate the differences in the type of movement. This could probably be caused by the reorganization of the cytoskeleton, but continued study is needed. The tested compound also inhibited the motility of melanoma cells, which have high metastatic ability, directly after its addition to the culture medium, causing a 50% decline in the average length of the cell trajectory value compared to the control. After 24 h pre-incubation with LTS-4, the value of the average cell displacement also decreased by about 50% compared to the control value (Tab. 3), while at this concentration, the influence of LTS-4 on the viability of melanoma cells was not so significant. Moreover, the motility of melanoma cells in the presence of LTS-4 and under control conditions was random – the value of the coefficient of movement efficacy (CME) was nearly zero (Tab. 3).

In conclusion, the results presented in this paper showed that saponoside LTS-4 significantly affected the viability, proliferation, morphology and cytoskeleton organisation of both cell lines. Our studies indicate that LTS-4 may also affect melanoma cell metastasis by the inhibition of cell motility. The most interesting result was that the tumour cells were more vulnerable to the tested compound than normal cells. Nevertheless, additional studies concerning the possible mechanism of action of LTS-4 are needed.

Acknowledgments. The authors would like to thank Professor Włodzimierz Korohoda and Professor Zbigniew Janeczko for their support and helpful

discussions. We would also like to thank Paulina Koczurkiewicz for her technical assistance.

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